

AD _____

AWARD NUMBER DAMD17-97-1-7145

TITLE: 99mTC Alpha-Fetoprotein: A Novel, Specific Agent for the
Detection of Human Breast Cancer

PRINCIPAL INVESTIGATOR: Bruce R. Line, M.D.

CONTRACTING ORGANIZATION: Albany Medical Center Hospital
Albany, New York 12208

REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
<small>Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.</small>				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 1998		3. REPORT TYPE AND DATES COVERED Annual (15 Jun 97 - 14 Jun 98)
4. TITLE AND SUBTITLE 99mTc Alpha-Fetoprotein: A Novel, Specific Agent for the Detection of Human Breast Cancer			5. FUNDING NUMBERS DAMD17-97-1-7145	
6. AUTHOR(S) Bruce R. Line, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Albany Medical Center Hospital Albany, New York 12208			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			19981210 083	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) <p>There is a pressing need for a widely available, non-invasive, and accurate imaging technique to further evaluate mammographically suspicious lesions to reduce the number of cancer negative biopsies. We have demonstrated that technetium-99m radiolabeled human alpha-fetoprotein (^{99m}Tc AFP) localizes in human breast cancer cells <i>in-vivo</i>, most likely concentrating in breast cancer cells due to a specific receptor not found in normal adult breast tissue. Our technical objective is to assess ^{99m}Tc AFP as an agent to detect and stage human breast cancer. As promised in the first two aims of the work scope, we have compared natural full-length and recombinant domain III preparations of ^{99m}Tc AFP to clinically used tracers, ²⁰¹Tl and ^{99m}Tc sestamibi, in immune-deficient (SCID) mice bearing human cancer xenografts. The results of these studies confirm our hypothesis that ^{99m}Tc AFP is a superior human breast cancer imaging agent. We have also demonstrated specific uptake of ^{99m}Tc AFP, <i>in-vitro</i>, and have measured the level of AFP receptors in the imaged tumors. During the second year of effort we will continue the work scope for these aims and also determine the ability of ^{99m}Tc AFP to image positive lymph nodes draining a transplantable rat mammary cancer.</p>				
14. SUBJECT TERMS Breast Cancer Alpha-fetoprotein, scintigraphic imaging, technetium-99m radiopharmaceutical, human tumor xenografts, nuclear medicine			15. NUMBER OF PAGES 22	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

X Where copyrighted material is quoted, permission has been obtained to use such material.

X Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

 In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Bruce R Line MD
PI - Signature

7/13/98
Date

Table of Contents

FRONT COVER	1
STANDARD FORM (SF) 298, REPORT DOCUMENTATION PAGE.....	2
FOREWORD	3
INTRODUCTION:.....	6
Subject of Research Work.....	6
Purpose of Investigation.....	6
Scope of Work.....	7
Task 1: Months 1-4: AFP production.	7
Task 2: Months 1-4: Grow human breast cancer cell lines as xenografts.....	7
Task 3: Months 3 & 4: Image MCF-7 and MDA-MB-231 breast cancer xenografts	7
Task 4: Months 5-10: Establish AFP receptor assay using MCF-7 and MDA-MB-231 breast cancer cell lines	7
Task 5: Months 5-24: Compare the imaging capability of ^{99m}Tc AFP to that of ^{99m}Tc sestamibi and ^{201}Tl thallium	7
Task 6: Months 5-24: Establish AFP receptor content in various tumors	8
Task 7: Months 12-24: Lymphoscintigraphy studies of ^{99m}Tc AFP in rat homograft model of mammary adenocarcinoma metastasis to lymph nodes.	8
Background of Prior Work.....	8
BODY OF ANNUAL REPORT	10
Experimental Methods, Assumptions and Procedures.....	10
Preparation of Recombinant Domain III AFP	10
Preparation of Natural human full length AFP from HepG2 cells.....	10
Xenograft Implantation Procedure	10
^{99m}Tc AFP Radiolabeling Procedure	11
Tracer Biodistribution and Imaging Studies	11
Assay for binding of ^{99m}Tc AFP to Tumor Cell AFP Receptors	12
Results and Discussion.....	13
Aim 1: Evaluate <i>in vivo</i> localization and biodistribution of ^{99m}Tc AFP candidates.	13
Aim 2: Evaluation of ^{99m}Tc AFP in a broad spectrum of tumors	13
Aim 3: Characterization of Cancer Cell AFP Receptors in-vitro.....	15
Aim 4: ^{99m}Tc AFP imaging of lymph nodes draining a primary rat mammary cancer.	16
Recommendations in Relation to the Statement of Work.....	16

SUMMARY & CONCLUSIONS18

REFERENCES19

APPENDICES22

INTRODUCTION:

Subject of Research Work

It is estimated that in 1995, in the United States, 182,000 new cases of breast cancer were diagnosed in women and 46,000 women died of the disease.(1) In addition to its cost of human lives, breast cancer consumes more health care dollars (\$6.5 billion) than any other cancer.(2) There is no question that early detection and removal of breast cancer is currently the best approach for curing this disease. Over 70% of US women in the age groups at risk for breast cancer have had at least one abnormal mammogram that detected a mass, a cluster of calcifications, or both, but these findings are not diagnostic of breast cancer. If mammographic studies are abnormal, either a breast biopsy is performed or the abnormality is reassessed with periodic follow-up mammograms until the nature of the abnormality is determined.(3) Unfortunately, mammography is far less sensitive and specific than the public perceives. Because of the lack of mammographic specificity, most biopsies of the breast are benign. In the US the probability of malignancy when a biopsy is performed on a mammographic abnormality usually ranges from 15-35%.(4-7) Consequently, for every woman who has a breast cancer detected by screening mammography, three to six women with false-positive results needlessly undergo the apprehension, pain and possible disfigurement associated with biopsy.

There is a pressing need for a widely available, non-invasive, and accurate imaging technique to further evaluate mammographically suspicious lesions to reduce the number of cancer negative biopsies. The need for a test with greater specificity than mammography also arises in patients with dense breast tissue, in patients with prior breast surgery, and in those who are at higher risk for breast cancer for whom the mammogram is equivocal. Recent studies using radionuclides have suggested that scintimammography has high sensitivity and improves the specificity of conventional mammography for the detection of carcinoma of the breast and thus, deserves study as a means to reduce the number of mammographically "indicated" biopsies of the breast that yield negative results for carcinoma. For the agents having the best results to date (^{99m}Tc Sestamibi, ^{201}Tl , ^{99m}Tc MDP, and ^{18}F -FDG), tumor uptake is related to nonspecific factors such as metabolic rate, capillary permeability, and membrane ion transport. These small molecules also migrate into background tissues and reduce potential sensitivity by clouding the scintigraphic image. In contrast, ^{99m}Tc AFP most likely concentrates in breast cancer cells due to a specific receptor not found in normal adult breast tissue. In our models, ^{99m}Tc AFP has greater specificity for imaging breast cancer than other agents currently in clinical trial.

The work we are carrying out focuses on the development of ^{99m}Tc alpha-fetoprotein (AFP) as a novel agent to detect and stage breast cancer.

Purpose of Investigation

Our hypothesis is that AFP is concentrated in breast cancer cells by a receptor-mediated process, thereby providing a novel means for detecting breast cancer cells specifically and with high sensitivity. The current studies will evaluate the potential for the clinical application of ^{99m}Tc AFP in patients with breast cancer. To test our hypothesis we are completing the following Specific Aims.

Aim 1. Among the three preparations of human AFP available to us (natural full-length, recombinant full-length, recombinant domain III), establish which one of these consistently yields the highest tumor-to-background ratios in immune-deficient (SCID) mice bearing either ER+ MCF-7 or ER-MDA MB 231 human breast cancer xenografts.

Aim 2. Using the preparation of choice established in Aim 1, determine the breadth of applicability of ^{99m}Tc AFP as an imaging agent for human breast cancers. This is accomplished by compar-

ing the imaging capability of ^{99m}Tc AFP to that of ^{201}Tl and ^{99m}Tc sestamibi in 12 different human tumor xenografts. Tumors consist of benign and malignant human breast tumor lines, non-breast tumor lines and freshly resected patient breast cancers.

Aim 3. Measure the level of AFP receptors in the imaged tumors as a potential explanation for the imaging capability of this protein.

Aim 4. Determine whether ^{99m}Tc AFP can image positive lymph nodes draining a transplantable rat mammary cancer.

Scope of Work

(abstracted from full work scope statement in original proposal)

Task 1: Months 1-4: AFP production.

Recombinant Domain III AFP: We produce Domain III of human AFP using a baculovirus vector which incorporates an N-terminal leader sequence from the baculoviral protein gp67, the Glutathione-S-Transferase protein from *Schistosoma japonicum* and Domain III of human AFP.

Natural human full length AFP: We purify human AFP secreted by HepG-2 cells using immunoaffinity chromatography, with purity and identity established by SDS-PAGE gels and Western Blots. We planned to purchase recombinant full length human AFP from Atlantic Biopharmaceuticals.

Task 2: Months 1-4: Grow human breast cancer cell lines as xenografts.

MCF-7 and MDA-MB-231 cell lines are expanded into multiple flasks, grown to confluence, harvested and centrifuged into cell pellets. Pellets are then solidified into fibrin clots, cut into pieces, and implanted into SCID mice.

Task 3: Months 3 & 4: Image MCF-7 and MDA-MB-231 breast cancer xenografts

Each of the AFP preparations is labeled with Tc-99m and injected i.v. into tumor xenograft-bearing mice. Mice are imaged and the ^{99m}Tc AFP preparations are assessed by their resultant tumor to thigh tissue ratios (T/Th) and clearance kinetics (% of injected dose/gram).

Task 4: Months 5-10: Establish AFP receptor assay using MCF-7 and MDA-MB-231 breast cancer cell lines

We proposed to radiolabel AFP with ^{125}I by the Chloramin T method and reproduce the studies reported by Uriel(8). We then wished to evaluate the AFP receptor content of MCF-7 and MDA-MB-231.

Task 5: Months 5-24: Compare the imaging capability of ^{99m}Tc AFP to that of ^{99m}Tc sestamibi and ^{201}Tl thallium

Each tumor line is expanded in culture and then transplanted into SCID mice. Replicate tumor-bearing mice are imaged in random order with ^{99m}Tc AFP, ^{99m}Tc sestamibi, and ^{201}Tl thallium. Biodistribution kinetics and tumor to thigh background ratios for each test agent is assessed. Studies are repeated for a variety of tumors.

Task 6: Months 5-24: Establish AFP receptor content in various tumors**Task 7: Months 12-24: Lymphoscintigraphy studies of ^{99m}Tc AFP in rat homograft model of mammary adenocarcinoma metastasis to lymph nodes.**

Obtain the 13762 NF rat mammary carcinoma cell line and transplant it into the dorsal region of the upper neck in female Fischer rats. The kinetics of metastasis to draining brachial and axillary nodes by necropsy and histopathology studies will be established. ^{99m}Tc AFP will be injected intravenously into tumor-bearing rats to image primary tumor and draining lymph nodes. ^{99m}Tc AFP will be injected s.c. in the region of the lymphatic drainage of the tumor to assess the potential advantage of regional administration of tracer for lymphoscintigraphy.

Background of Prior Work

Current methods of detecting breast cancer have low specificity and sensitivity. Although screening mammography results in early detection of breast cancer and reduces death from this disease, it has a low positive predictive value and a 60-90% false positive rate that leads to the pain, morbidity, and potential disfigurement associated with an estimated 500,000 unnecessary breast biopsies. Furthermore, true positive mammography is not helpful in assessing prognosis or predicting therapeutic response. Scintigraphic methods to detect breast cancer offer a means to improve the evaluation of patients with positive breast exams or positive mammograms.

^{201}Tl and ^{99m}Tc sestamibi are currently in clinical trial as imaging agents for breast cancer. Although early results have shown some promise (9-11), improvements in both sensitivity and specificity are needed. For example, a recent large multicenter trial enrolled 673 patients included 377 women with non-palpable mammographically detected abnormalities. The overall sensitivity and specificity for ^{99m}Tc sestamibi was 85% and 81% respectively with 72% sensitivity and 86% specificity for non-palpable tumors(12). False positive sestamibi studies have been found in cases of fibrocystic disease and in fibroadenomas. These false positive results may be due to hypercellularity and proliferative changes(13) or to the non-specific "metabolic activity" of these lesions.(10) Furthermore, ^{99m}Tc sestamibi is eliminated from the cell by the multidrug resistance P-glycoprotein (gp170) which is over expressed in some breast cancers.(14) Finally, ^{201}Tl and ^{99m}Tc sestamibi both produce images with variable patterns of normal breast activity that is in part due to significant scatter from non-specific uptake in cardiac and abdominal tissues.(15)

Alpha-fetoprotein is a serum protein produced by fetal liver and crosses into the maternal circulation during pregnancy. Although developing tissues have the ability to bind and endocytose AFP, this function is lost by adult differentiated cells (8,16,17). The capability reappears, however, in neoplastic cells growing either *in vivo* (18) or *in vitro* (8,19,20). Malignant cells that have been shown to take up AFP include human breast cancer cells, malignant lymphoblastoid cells, neuroblastoma cells, and rhabdomyosarcoma cells (8,21,22). Our collaborative "AFP group" has studied AFP and Domain III of human AFP because of their properties as inhibitors of breast cancer growth.(23-27) We have found that ^{99m}Tc radiolabeled recombinant human AFP (^{99m}Tc AFP) localizes rapidly and specifically in human breast cancer xenografts, providing well-defined images of the tumor relative to normal tissues.(28) Our preliminary studies indicate that ^{99m}Tc radiolabeled human AFP and domain III of human AFP show substantially better localization in human breast tumor xenografts than either ^{201}Tl or ^{99m}Tc sestamibi.(28,29) Furthermore, ^{99m}Tc AFP localizes in both estrogen receptor positive (ER+) MCF-7 and ER- MDA MB 231 breast tumors and has blood clearance through the kidneys. These features are critically important to an agent that must have high specificity.

Although scintimammography using non-specific tracers such as ^{99m}Tc sestamibi can improve the specificity of diagnostic imaging, it is likely that further gains in both specificity and sensitivity can be achieved with AFP because it is selectively taken up by breast cancer and has low non-specific uptake in normal tissues. The fact that our preliminary results indicate that ^{99m}Tc AFP demonstrates greater image specificity for breast cancer than either ^{201}Tl or ^{99m}Tc sestamibi suggests strongly that ^{99m}Tc AFP should be developed further for this purpose.

Scintimammography should also be further explored as a staging technique for breast cancer. Metastasis to the axillary lymph nodes is an important prognostic factor. Patients with pathologically negative axillary lymph nodes have a recurrence rate of 20-40% over a 10-year period (30,31), while those with one to three positive nodes have a recurrence rate of almost twice that (32). Axillary lymph node sampling requires an incision separate from the primary surgical procedure, adding to the morbidity and cost of treatment of primary breast cancer. Therefore, a noninvasive technique to evaluate axillary lymph nodes should be safer and more economical. Unfortunately, although quite safe, the low relative sensitivity of radiocolloid axillary lymphoscintigraphy (60-70%), ^{99m}Tc sestamibi (47-79%), and ^{201}Tl imaging (57%) compared to surgical axillary evaluation, have not led to their wide use in clinical practice (9,33-36). To achieve higher accuracy, it is likely that a tracer that specifically concentrates in tumor cells will be required (37). We plan to assess the ability of ^{99m}Tc AFP to detect spontaneous metastases in lymph nodes draining the site of a primary breast cancer. This will be evaluated in the transplantable 13762 NF rat mammary adenocarcinoma model, which reliably metastasizes to draining lymph nodes within two weeks after transplantation into syngeneic rats (38).

BODY OF ANNUAL REPORT

Experimental Methods, Assumptions and Procedures

Preparation of Recombinant Domain III AFP

This was produced using a baculovirus vector (pACSecG2T, Pharmingen) which incorporated an N-terminal leader sequence from the baculoviral protein gp67 (to facilitate secretion from insect cells), the Glutathione-S-Transferase (GST) protein from *Schistosoma japonicum* (to facilitate purification and solubilization of fusion protein) and Domain III of human AFP. The transfer vector containing the coding sequence for Domain III of hAFP was cotransfected into SF9 insect cells to produce recombinant virus. Virus was plaque-purified, then screened for the incorporation of the Domain III coding sequence into the viral genome (by PCR; Polymerase Chain Reaction) and for the ability of recombinant virus to produce secreted protein (Western Blot).

Recombinant baculovirus containing the cDNA for Domain III (previously described) was amplified by three serial passages and titered by plaque assay in order to produce a large quantity of virus. Protein was then produced in large batches by infection of SF9 cells with recombinant virus. The medium containing the secreted Domain III protein is harvested and clarified by centrifugation to remove cell debris which results from cell lysis. The protein is purified by loading the clarified cell culture medium onto a Glutathione-Agrose (Sigma) column. The column was washed with PBS and then treated with Thrombin (Sigma) to release the Domain III fragment. The thrombin released fragment will be identified by Western Blot analysis using polyclonal antibody to human AFP and silver-stained SDS-PAGE. Protein was then aliquoted (50µg/each) and stored lyophilized at -80°C.

Preparation of Natural human full length AFP from HepG2 cells

Culture conditions for HepG2 cells. HepG2 cells were maintained and grown as a monolayer in α MEM (GIBCO, Grand Island, NY) supplemented with 5% serum (2/5 calf serum, 3/5 fetal calf serum), penicillin G (100 units/ml), and streptomycin (100 µg/ml). Cells were released from monolayer using 0.25% trypsin/0.25% EDTA. Subculturing into additional flasks was carried out by five-fold dilution of cells in the above maintenance medium. Confluent flasks were switched to serum-free medium to up-regulate production of AFP as described by Tecce et al. (17). Serum-free medium is comprised of 3 parts α MEM: 1 part Waymouth's MB 752/1 plus 3×10^{-8} M sodium selenite, 2 mM L-glutamine and 1.5% antibiotic/antimycotic mixture from GIBCO. Cells were refed with serum-free medium every three days.

Purification of AFP from Culture Supernatants of HepG2 Cells. HepG2 culture supernatants were pooled and concentrated using P-10 Centriprep concentrators (Amicon, Beverly, MA). Ten ml of concentrate containing approximately 3 mg of AFP were loaded onto an 18 cm x 2.5 cm immunoaffinity column (rabbit anti-human AFP (DAKO) conjugated to cyanogen bromide-activated Sepharose 4B) in a loading buffer of 100 mM NaCl/10 mM sodium phosphate pH 7.4. Concentrate was incubated on the column at room temperature for 30 minutes. Non-AFP proteins were eluted with approximately 200 ml loading buffer until no protein was detectable in the eluate by UV absorbance (280 nm). AFP is eluted with approximately 200 ml of 1.8 M $MgCl_2$ and dialyzed immediately against excess 10mM sodium phosphate buffer, pH 7.2. This material was washed and concentrated in a buffer comprised of 100 mM sodium chloride-10 mM sodium phosphate, pH 7.2.

Xenograft Implantation Procedure

For xenograft implantation, human tumor cell lines were grown to confluence and harvested by trypsinization. Cells were converted to solid tumor form by centrifugation into a pellet and exposure

of the cell pellet to 15 μ l of fibrinogen (50 mg/ml) and 10 μ l of thrombin (50 units/ml) for 30 minutes at 37°C. Fibrin clots were cut into pieces approximately 1.5 mm in diameter. Six to eight pieces were loaded into a 16 gauge trocar and were implanted subcutaneously in the region of the brachial lymph node near the front limb of female CB17 SCID mice. Tumors were usually palpable 3 to 4 weeks after implantation and reached a diameter of 1 cm approximately 6 weeks after implantation. To assess tracer sensitivity, imaging studies were performed when tumors were first palpable, approximately 0.2 cm in diameter, and as they enlarge to 1 cm in size.^(25,39,40)

^{99m}Tc AFP Radiolabeling Procedure

^{99m}Tc labeling of AFP was carried out via stannous ion reduction as previously described (41). Briefly, Tc^{99m} AFP was prepared from a 50 μ g AFP aliquot mixed with .5ml 0.9% Sodium Chloride Injection (Baxter Healthcare). The solution was added to an Ultra Tag RBC™ Reaction Vial (Mallinckrodt Medical Inc., St. Louis, MO), and the contents of the vial were mixed by gentle swirling, and incubated at room temperature for 5 minutes. At the completion of the incubation time, 20-1000 MBq ^{99m}Tc Sodium Pertechnetate Injection (Mallinckrodt Medical, Inc., St. Louis, MO) was added in a volume of 1-2 ml. The contents of the vial were mixed by gentle swirling and were incubated for 15 minutes. Dose aliquots were assayed using thin-layer chromatography performed on preparation using ITLC-SG (Gelman Instrument Co., Ann Arbor, MI) with acetone. Typically, 92 - 100% of the ^{99m}Tc was bound to AFP. The preparation was not used in studies if the percent bound was less than 90%.

Tracer Biodistribution and Imaging Studies

In vivo biodistribution data was collected in up to 6 mice imaged simultaneously on a Siemens gamma camera. The data was collected by a dedicated computer and transferred to a Pentium PC MS Windows-based image processing system for analysis.^(42,43) After sedation with intraperitoneal administration of 50 mg/kg pentobarbital, the mice were injected intravenously with 20-40 MBq of the tracer compound (^{99m}Tc AFP (3 μ g), ^{99m}Tc Sestamibi, or ²⁰¹Tl) and then were placed in the prone position on a thin polyethylene panel. To eliminate motion during imaging, the mice were restrained on panels by strips of tape over their extremities so as not to restrict respiration. Dynamic images obtained over 60 minutes were used to determine the biodistribution of the labeled agent. Typically, twelve sequential, five minute images were obtained with low energy general purpose collimation, and 1.5 hardware zoom into computer matrices having 128 by 128 picture elements.⁽⁴²⁾ Images were analyzed by positioning "regions of interest" (ROI) over the tumor, thigh, contralateral chest wall and total body. The counts were corrected for radioisotope decay to facilitate activity comparisons.

The image data were evaluated to determine the activity in the tumor tissue by drawing a region of interest that included all margins of the tumor. Where the tumors were not visibly apparent, regions were placed over the area where the tumor was palpated at the time of imaging. A second region was placed over the contralateral chest wall by reflecting the tumor region of interest through the midline axis. The second region was used to define a background activity that was subtracted from the tumor region to yield counts in the tumor tissue. The gram weight of the tumor was determined from a volume calculation based on measured tumor diameter. A third region of interest was defined over the thigh and the counts per gram of thigh tissue was obtained by using a thigh mass of 6% of body weight. A fourth region of interest was defined over the entire animal to determine the total injected activity. Regions were produced for each animal study at both 1 and 24 hour image collection points. Tracer localization parameters were determined by 1) the tumor uptake as the percent of injected activity per gram of tissue (%ID/gram); and 2) the tumor to thigh tissue background (T/Th) ratio determined from the ratio of tumor activity per gram to the thigh activity per gram.

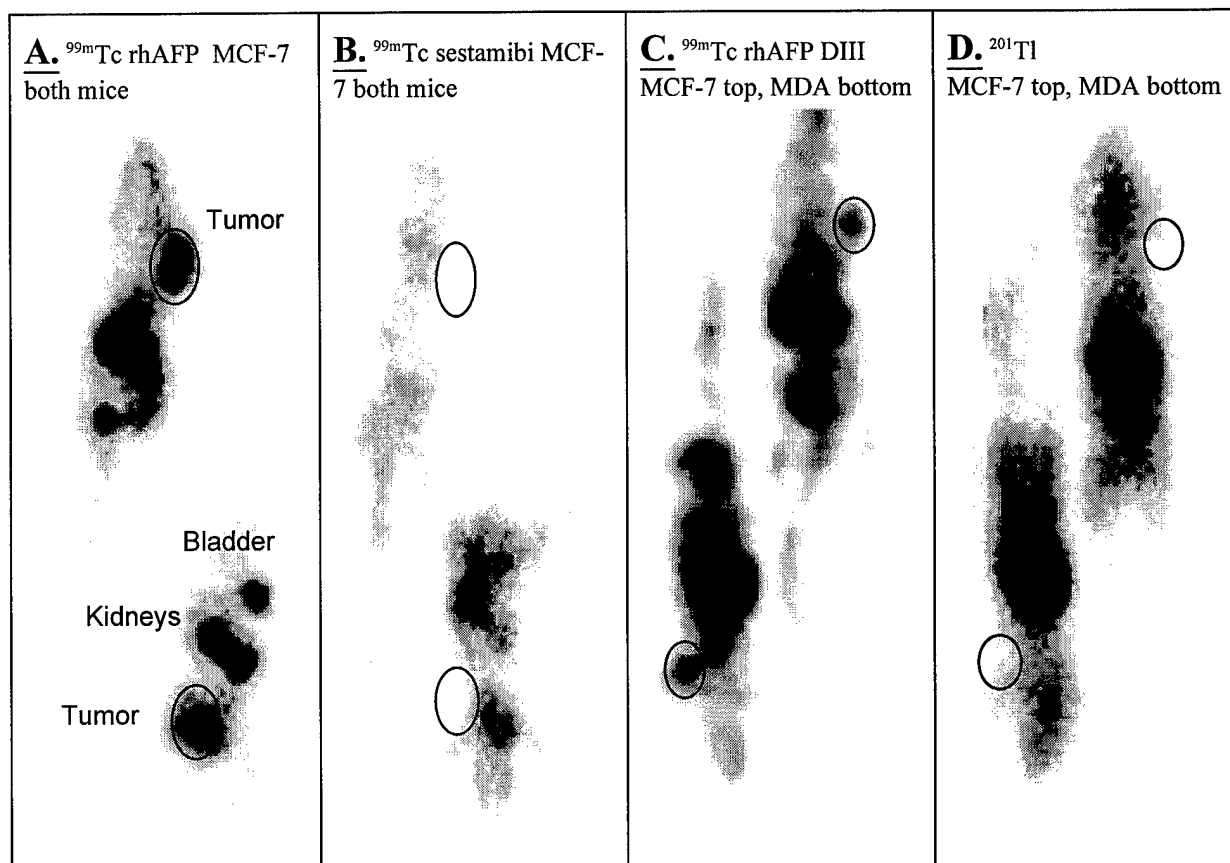


Figure 1: Sequential studies in mice bearing human tumor xenografts.

Panels A, B: 2 mice bearing MCF-7 ER+ human breast cancer xenografts 24 hours after doses of 37mbq of ^{99m}Tc AFP (A) and ^{99m}Tc sestamibi (B). Studies in panel A were performed 7 days after those in panel B in the same animals. Over this interval, tumors in right infra-axillary region were 1.2 cm in diameter. (about 900 mg)

Panels C, D: 2 mice bearing xenografts (top: MCF-7, bottom MDA MB-231 ER- tumor) 24 hours after doses of 37mbq of ^{99m}Tc AFP Domain III (C) and ^{201}Tl (D) Tumors in right infra-axillary region were 0.6cm in diameter (about 120 mg). Studies were 7 days apart (D after C) in the same animals.

Assay for binding of ^{99m}Tc AFP to Tumor Cell AFP Receptors

An assay for AFP receptor was developed by our group. The published procedure using radioiodinated AFP utilizes prohibitively large amounts of cold AFP in incubations to block receptors so that non-specific binding of the radio-iodinated material can be evaluated(8). Therefore, a technique that did not require such a step was needed. We took advantage of the exquisite sensitivity of the Abbott IMx immuno-quantitation of AFP (0.2 ng/ml) and the published report that AFP is dissociated from its receptor in 0.4 M KCl (44). Replicate tubes containing 2.5×10^6 cells in 0.2 ml serum-free medium were incubated with varying concentrations of AFP for 3 hours at 4°C. Cells were washed four times by centrifugation and resuspension in serum-free medium. After the final washing there was no detectable AFP in the supernatant (i.e., less than 0.2 ng/ml, indicating virtually complete removal of AFP from lower affinity non-specific sites). Sodium azide (20 mM, 5 min, 4°C) was then added to prevent receptor-ligand complexes on the cell membranes from internalizing when cells were subsequently warmed. KCl (0.4 M final concentration) was then added and incubated for one hour at 37°C to dissociate AFP-AFP receptor complexes. Cells were then centrifuged at 2,000 rpm

for 10 minutes, supernatant was removed and the KCL liberated AFP content of the supernatant was determined. By Scatchard plot analysis of AFP bound at different incubation concentrations, the number of specific binding sites per cell and their binding affinity were determined. Binding of AFP increased with increasing concentration and plateaued at 30 ng/ml. Bound AFP was approximately 0.1% of total AFP added to the cells. Therefore, concentration of free AFP was assumed to be equal to that of total AFP.

Results and Discussion

Aim 1: Evaluate *in vivo* localization and biodistribution of ^{99m}Tc AFP candidates.

The information from the preliminary studies we have carried out suggests that Tc-99m AFP will be an excellent imaging agent for human breast cancer. The studies of aim 1 were designed to allow us to compare the candidate Tc-99m AFP molecules in two human breast cancer xenografts (MCF7 an estrogen receptor positive (ER+) tumor and MDA MB 231, an ER- tumor cell line). The purpose of this aim was to identify which of the available ^{99m}Tc AFP molecules had the best tumor localization and imaging performance. This compound was to then be further tested in comparison with ^{99m}Tc Sestamibi and ^{201}Tl in a broad range of tumors (table 1). Image examples of the localization of the radiopharmaceuticals are shown in figure 1 panels A and B. The quantitative comparisons of tumor percent injected dose/gram (%ID/gram) and tumor to thigh ratio (T/Th) are shown in the appendix in table 3. The results of the studies have been summarized using standard descriptive statistics. Comparison of differences between the different preparations have also been analyzed using analysis of variance with pair-wise comparisons done by Student-Newman-Keuls method.

We initially planned to compare ^{99m}Tc radiolabeled natural full length AFP, recombinant full length AFP and recombinant domain III of AFP (DIII). Unfortunately, the supply of recombinant full length AFP from Atlantic Biopharmaceuticals was interrupted by financial instability and subsequent failure of that corporation. Our studies with the two remaining forms of AFP showed better performance of full length natural AFP in the MCF7 xenografts whereas the DIII radiopharmaceutical appeared superior in the MDA MB 231 xenografts (see table 3). Given the reduced number of candidate AFP compounds and the uncertainty as to which of the remaining would prove to be superior, we decided to test both the full length AFP and DIII in the tumors (see table 1) studied in Aim 2.

Aim 2: Evaluation of ^{99m}Tc AFP in a broad spectrum of tumors

We evaluated the ^{99m}Tc AFP preparations by comparison to two other clinically available tracers that are being evaluated in patients with breast cancer: ^{99m}Tc sestamibi and ^{201}Tl . The tumor tissues represent breast and non-breast cancers that were steroid receptor positive or steroid receptor negative. Replicate tumor xenograft-bearing mice were used to assess the imaging capability of ^{99m}Tc full length AFP, ^{99m}Tc DIII, ^{99m}Tc sestamibi, and ^{201}Tl for each tumor. Four imaging studies were performed in a set of six replicate tumor xenograft-bearing mice, one for each tracer. The studies were spaced by 3-5 days to allow the previous tracer to decay and to allow the animals to fully re-equilibrate. The sequence of tracer administration was random. The tumor size and appearance was recorded over the interval to correlate size with detectability and tumor to thigh tissue ratio. The completion status of the tumor studies is shown in the second column of table 1.

The data were analyzed to address three specific questions. 1] Is radioactivity in tumor significantly above that in background tissue? 2] Is radioactivity in tumor from ^{99m}Tc AFP significantly above that from ^{99m}Tc sestamibi or ^{201}Tl ? 3] Are there differences among tumors in radioactivity from ^{99m}Tc AFP?

Breast Tumor Designation	Status	Histological and Biological Description
MCF-7	Tested	ER+ breast cancers inhibited by Tamoxifen
T-47D	Tested	ER+ breast cancers inhibited by Tamoxifen
MCF-7/TAM	Planned	ER+ clone of MCF-7 resistant to Tamoxifen
MDA MB 231	Tested	ER- breast cancers not inhibited by Tamoxifen
BT-20	Tested	ER- breast cancers not inhibited by Tamoxifen
Freshly Resected Patient Breast Cancer	Growing	ER+ primary carcinoma of the breast
Non-Breast Tumors		
EnCa 101	Planned	ER+ endometrial cancer
NIH: OV-CAR-3	Planned	ER+ ovarian cancer
MFE	Tested	Endometrial
MTW9A	Tested	Rat mammary tumor
LnCaP	Tested	AR+ prostate cancer
DU-145	Tested	AR- prostate cancer
NCI-H520	Planned	Squamous cell carcinoma of the lung
Other Breast Tissues		
MCF-10A	Planned	Non-cancerous cell line established from a patient with fibrocystic breast disease.
Hs-578Bst	Planned	Normal breast myoepithelial cell line. The tissue from which this line was isolated was adjacent to an infiltrating ductal carcinoma which was the source of the Hs-578T cell line

Table 1: Cell lines considered for testing in aim 2. Cell lines include breast cancer, non-breast tumor, and non-tumor tissues. Each line is to be grown as a xenograft in SCID mice. Where successful the xenografts will be used in studies which compare the tumor-imaging capability of ^{99m}Tc AFP to that of ^{99m}Tc Sestamibi and ^{201}Tl . (*ER = estrogen receptor, AR = androgen receptor)

To evaluate whether the activity in tumor is significantly greater than thigh tissue activity, we compared the counts per gram in tumor to the counts per gram in thigh tissue (T/Th). This evaluation was performed for all tumors greater than 0.25 grams. Tumors smaller than this were excluded because they were difficult to locate in the images reliably. Figures 2A, 2B (Table 3A, 3B) show the T/Th results at 1 and 24 hours respectively. Values greater than one indicate a higher tumor localization than thigh tissue activity. (It should be noted that a T/Th parameter is used to give a more comparable measure than is available from the usual tumor to thigh tissue background measures obtained from imaging studies. The tumor region was not compared to the contralateral chest areas because of the high variability of the ratio with small changes in region placement.)

For most of the tumors, the T/Th ratios are greater than 1 for both AFP and DIII, where all of the ratios are less than 1 for ^{99m}Tc Sestamibi and ^{201}Tl . Analysis of variance indicates that in most comparisons the T/Th measures are significantly greater for AFP and DIII relative to ^{99m}Tc sestamibi and ^{201}Tl . It is of interest that for some tumors (LNCaP, MCF7), AFP shows the best T/Th perform-

Figure 2A

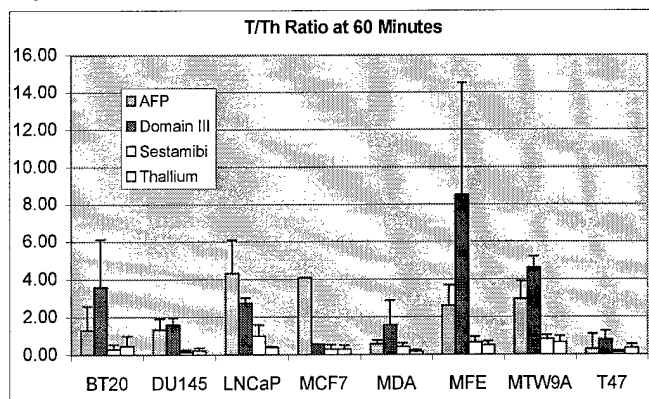


Figure 2B

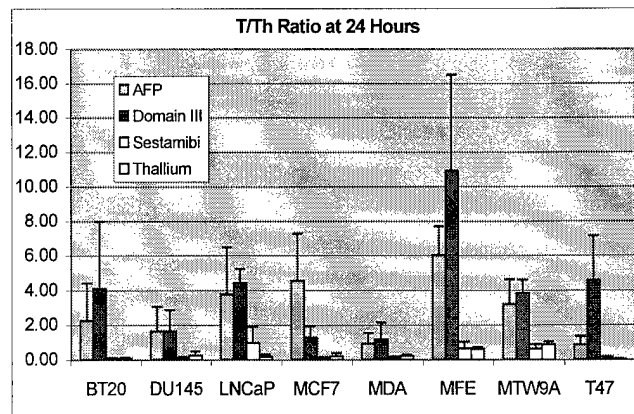


Figure 2C

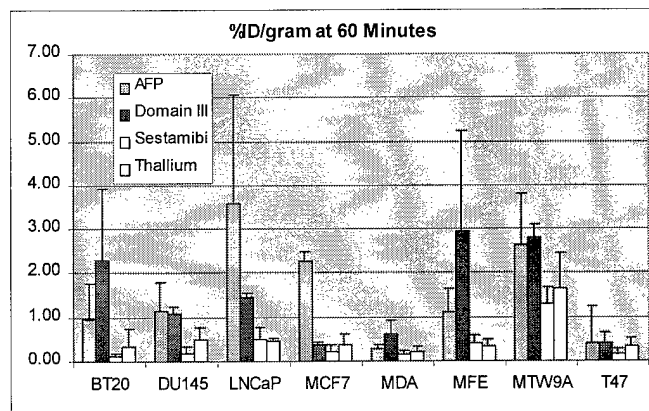


Figure 2D

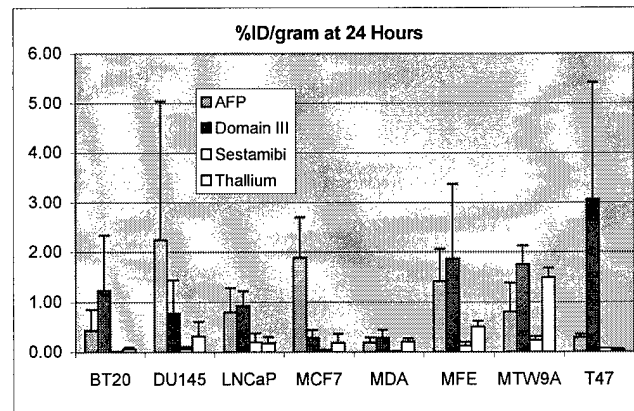


Figure 2: Mean and standard deviation of tumor localization measures at 1 and 24 hours.

ance where as DIII is superior in most of the other tumors tested thus far. In all breast cancers (BT20, MCF7, MDA, MTW9A, T47), T/Th increases from 1 to 24 hours with ^{99m}Tc full length AFP suggesting good tumor retention of this radiopharmaceutical. However, in 4 of 5 breast cancers, the T/Th ratio was higher with ^{99m}Tc DIII than with ^{99m}Tc full length AFP. It would thus seem reasonable to continue testing both of the AFP preparations in the remaining tumor cell lines to be studied in year 2.

By analysis of variance of the percent injected dose/gram of tumor tissue data, significant differences in tumor localization exist between the tested radiopharmaceuticals. At one hour for example, for BT20, ^{99m}Tc DIII was significantly higher than ^{99m}Tc AFP, ^{99m}Tc Sestamibi and ^{201}Tl . Whereas, for MCF7, ^{99m}Tc full length AFP was significantly higher than all other radiopharmaceuticals. Inspection of figures 2C, 2D (Table 3C, 3D) indicates that for most tumors, the %ID/gram uptake for ^{99m}Tc AFP and ^{99m}Tc DIII is significantly higher than ^{99m}Tc Sestamibi or ^{201}Tl .

Thus, in the studies performed to date, ^{99m}Tc AFP and ^{99m}Tc DIII appear to offer significant imaging advantages in a wide range of tumor cell lines with regard to tumor uptake and relative contrast to normal tissue.

Aim 3: Characterization of Cancer Cell AFP Receptors in-vitro

All of the tumors studied thus far contain high affinity binding sites for ^{99m}Tc AFP. The number of binding sites per cell is found to be consistent with cells that have high affinity receptors for ligand. Table 2 shows the results of the binding affinity and number of receptors per cell for four tumor cell lines tested to date. The highest affinity was found for the T47 tumor and the MDA MB 231

cell line had the greatest number of receptors per cell. There appears to be a wide range of receptor affinities for the ^{99m}Tc AFP radiopharmaceutical. (nearly 2 orders of magnitude).

As outlined in our original proposal, we compared the in-vitro receptor data to the in-vivo imaging data. The number of sites per cell did not correlate with the percent of injected dose per gram tumor either at 1 hour or at 24 hours. However, it is of interest that the T47 cell line had the highest binding affinity for ^{99m}Tc AFP and it was also that tumor which had the highest increase in T/Th ratio over a 24-hour period. The high binding affinity may be an explanation for the high *in-vivo* retention and this will be studied further in the second year of the project.

Table 2: AFP Receptor Binding Study Results				
TUMOR (P:prostate, B: breast)	MDA B / ER-	MCF 7 B / ER+	T 47 B / ER+	LNCaP P / AR+
AFP Binding Kd	6×10^{-8}	3.8×10^{-8}	1.8×10^{-10}	3.9×10^{-9}
Receptors - #/cell	140,000	43,800	30,200	31,000

Aim 4: ^{99m}Tc AFP imaging of lymph nodes draining a primary rat mammary cancer.

The 13762 NF rat mammary adenocarcinoma is a transplantable tumor which, following implantation into either the mammary fat pads, or into subcutaneous or intracutaneous site, spontaneously metastasizes to the regional lymph nodes and then to the lungs.(38) In addition to assessing the ability of ^{99m}Tc AFP to image regional lymph nodes, the rat model enables us to address the question as to whether subcutaneous administration near the primary tumor site may be more effective than intravenous administration for imaging tumor in regional lymph nodes.

This portion of the work scope is to take place in the next year of the project, thus no results have been obtained.

Recommendations in Relation to the Statement of Work

Task 1: Months 1-4: AFP production.

This task has been completed for both recombinant Domain III AFP and natural human full length AFP. Sufficient material is now available for completion of the studies proposed. The process described for production of natural human full length AFP is ongoing as this material is needed in large quantities for other projects. The plans to purchase full length recombinant AFP from Atlantic Biopharmaceuticals were frustrated by the decision of the supplier to discontinue production. No further work with this currently unavailable source is planned.

Task 2: Months 1-4: Grow human breast cancer cell lines as xenografts.

MCF-7 and MDA-MB-231 cell lines have been expanded, grown, harvested and implanted into SCID mice. The task is completed as proposed. In addition other cell lines have been implanted as xenografts as described in the results and discussion section.

Task 3: Months 3 & 4: Image MCF-7 and MDA-MB-231 breast cancer xenografts

Each of the AFP preparations (recombinant Domain III of human AFP and natural full length AFP) was labeled with Tc-99m and injected i.v. into tumor xenograft-bearing mice, completing the task as planned. Mice were imaged and the ^{99m}Tc AFP preparations were assessed by their resultant tumor to thigh tissue background ratios and clearance kinetics. Although we initially planned to focus on the best agent for further study (task 5), our initial results showed that both agents worked well

and that there was not a clearly superior agent. We therefore have proceeded to test both agents in xenografts of other tumors and will continue to do so in order to characterize the relative performance of the agents. Eight different tumors were imaged during year 1 and an additional four tumors, including freshly resected patient cancers will be imaged during year 2.

Task 4: Months 5-10: Establish AFP receptor assay using MCF-7 and MDA-MB-231 breast cancer cell lines

We proposed to radiolabel AFP with 125I by the Chloramin T method and reproduce the studies reported by Uriel(8). We then wished to evaluate the AFP receptor content of MDA-MB-231. This proved not to be feasible for the reasons discussed in the results and discussion section. We have substituted a procedure that we have characterized and propose to use to complete the work. The overall purpose of the task has been achieved albeit by a different route than originally planned. This task is therefore considered complete.

Task 5: Months 5-24: Compare the imaging capability of 99mTc AFP to that of 99mTc sestamibi and 201 thallium

We have studied 8 tumor lines to date of the 12 originally proposed. We have completed an initial analysis of the data and have found that the results support the hypothesis that AFP is concentrated in breast cancer cells, thereby providing a novel means for detecting breast cancer cells specifically and with high sensitivity. Moreover, the results indicate that 99mTc AFP was a better imaging agent than 99mTc sestamibi and 201thallium in all of the tumors studied during the first year of the project. The results to date suggest that the methodology is satisfactory and that the data collection should be completed as planned over the next 12 months of the project.

Task 6: Months 5-24: Establish AFP receptor content in various tumors

The progress to date in this task has been slowed by methodological detours and revisions. We have now developed satisfactory procedures that should allow the remainder of the data to be collected within the planned time frame for this task. Four tumor cell lines have been analyzed and the remaining tumors will be evaluated in the second year of the project.

Task 7: Months 12-24: Lymphoscintigraphy studies of 99mTc AFP in rat homograft model of mammary adenocarcinoma metastasis to lymph nodes.

We have yet to begin this task except for planning. We will obtain the 13762 NF rat mammary carcinoma cell line and transplant it into the dorsal region of the upper neck in female Fischer rats. The kinetics of metastasis to draining brachial and axillary nodes by necropsy and histopathology studies will be established. 99mTc AFP will be injected intravenously into tumor-bearing rats to image primary tumor and draining lymph nodes. 99mTc AFP will be injected s.c. in the region of the lymphatic drainage of the tumor to assess the potential advantage of regional administration of tracer for lymphoscintigraphy. We expect that the task will be completed as planned over the next 6-9 months.

SUMMARY & CONCLUSIONS

Current methods of detecting breast and prostate cancer have low specificity and sensitivity. Receptors for AFP have been detected on many malignant cells but are not expressed by normal tissue cells. We studied the binding affinity of AFP for human breast and prostate cancer cell lines (Scatchard Analysis) and evaluated the imaging characteristics of ^{99m}Tc full length AFP (AFP) and ^{99m}Tc recombinant human AFP Domain III (DIII) relative to ^{99m}Tc sestamibi and ^{201}Tl . Studies were carried out using human tumor cell lines from estrogen receptor (ER) positive (MCF7, T47) and ER negative (MDA-MB 231) human breast cancers as well as androgen receptor (AR) positive (LNCaP) and AR negative (DU 145) prostate cancers. Tumor xenografts were placed in the lateral thorax region of CB-17 SCID mice and grown to a size of approximately 0.8-2.0 cm diameter (0.27-4 gm). Tracer kinetics were measured at 0-60 minutes and at 24 hours following injection of 37MBq of either ^{99m}Tc AFP (4-6 μg), ^{99m}Tc DIII (4-6 μg), ^{99m}Tc sestamibi or ^{201}Tl .

Our results suggest that breast and prostate tumors demonstrate high affinity receptors for AFP. ^{99m}Tc AFP and ^{99m}Tc DIII specifically localize in human breast and prostate cancers in vivo with higher target to background localization than either ^{99m}Tc sestamibi or ^{201}Tl . ^{99m}Tc AFP and ^{99m}Tc DIII show promise as imaging agents for the detection and staging of breast and prostate cancer.

REFERENCES

1. Wingo PA, Tong T, Bolden S. Cancer Statistics: 1995. *CA Cancer J Clin* 1995; 45:8-30.
2. Ziegler J. New database allows researchers to evaluate cancer care costs. *J Natl Cancer Inst* 1993; 85:351.
3. McKenna RJ Sr. The Abnormal Mammogram Radiographic Findings, Diagnostic Options, Pathology, and Stage of Cancer Diagnosis. *Cancer Supplement* 1994 74: 244-255.
4. Bird RE, Wallace TW, Yankaskas BC,. Analysis of cancers missed at the screening mammography. *Radiology* 1992; 184: 613-617.
5. Yankaskas BC, Knelson MH, Abernathy ML. Needle localization biopsy of occult lesions of the breast: experience in 199 cases. *Invest Radiol* 1988; 23: 727-733.
6. Rosenberg AL, Schwartz GF, Feig SA. Clinically occult breast lesions: localization and significance. *Radiology* 1987; 162:167-170.
7. Meyer JE, Eberline TJ, Stomper PC, Sonnefeld MR. Biopsy of occult breast lesions: Analysis of 1,261 abnormalities. *JAMA* 1990; 263: 2341-2343.
8. Villacampa MJ, Moro R, Naval J, Failly-Crepin C, Lampreave F, Uriel J. Alpha-fetoprotein receptors in a human breast cancer cell line. *Biochem Biophys Res Commun* 1984 122: 1322-1327.
9. Waxman AD, Ramanna L, Memsic LD, Foster CE, Silberman AW, Gleischman SH, Brenner RJ, Brachman MB, Kuhar CJ, Yadegar J. Thallium scintigraphy in the evaluation of mass abnormalities of the breast. *J Nucl Med* 1993; 34: 18-23.
10. Khalkhali I, Mena I, Jouanne E, Diggles L, Venegas R, Block J, Alle K, Klein S. Prone scintimammography in patients with suspicion of carcinoma of the breast. *J Am Coll Surg* 1994; 178: 491-7.
11. Taillefer R, Robidoux A, Turpin S, Laperriere J. 99mTc-sestamibi prone scintimammography in detection of primary breast cancer and axillary lymph node involvement. *J Nucl Med* 1995; 36:52P.
12. Khalkhali I, Villanueva-Meyer J, Edell SL, Hanelin IG, Lugo CE, Taillefer R, Freeman-L M, Neal CE, Sheff AM, Connolly JL, Schnitt SJ, Baum JK, Houlihan MJ, Hale CA, Haber SB,. Diagnostic Accuracy of Tc-99m Sestamibi Breast Imaging in Breast Cancer Detection. *J Nucl Med* 1996; 37: 74P.
13. Gupta P, Waxman A, Nguyen K, Phillips E, Yadegar J, Silberman A, Memsic L. Correlation of Tc-99m Sestamibi Uptake with Histopathologic Characteristics in Patients with Benign Breast Disease. *J Nucl Med* 1996; 37: 250P.
14. Ciarmiello A, Del Vecchio S, Potena MI, Mainolfi C, Carriero T, Tsuruo T, Marone A, Salvatore M. Tc-99m Sestamibi Efflux and P-Glycoprotein Expression in Human Breast Carcinoma. *J Nucl Med* 1995; 36: 129P.
15. Maurer AH, Caroline FJ, Manzone TA, Maier WP, Au FC, Schnall SF. Limitations of Cranio-caudal Thallium-201 and Technetium-99m Sestamibi Mammoscintigraphy *J Nucl Med* 1995; 36:1696-1700.
16. Trojan J, Uriel J. Immunocytochemical Localization of Alpha-Fetoprotein (AFP) and Serum Albumin (ALB) in Ecto-, Meso-, and Endodermal Derivates of the Developing Rat. *Oncodevel Biol Med* 1982 3: 13-22.
17. Laborda J, Naval J, Calvo M, Lampreave F, Uriel J. Alpha-Fetoprotein and Albumin Uptake by Mouse Tissues During Development. *Biol Neonate* 1989 56: 332-341.

18. Uriel J, Villacampa MJ, Moro R, Naval J, Faily-Crepin C. Uptake of Radiolabeled Alpha-Fetoprotein by Mouse Carcinomas and its Usefulness in Tumor Scintigraphy. *Cancer Res* 1984 44: 5314-5319.
19. Geuskens M, Naval J, Uriel J. Ultrastructural Studies of the Intracellular Translocation of Endocytosed Alpha-Fetoprotein (AFP) by Cytochemistry and of the Uptake of ³H-Arachidonic Acid Bound to AFP by Autoradiography in Rat Rhabdomyosarcoma Cells. *J cell Physiol* 1986 128: 389-396.
20. Uriel J, Poupon MF, Geuskens M. Alpha-Fetoprotein Uptake by Cloned Cell Lines Derived from a Nickel-induced Rat Rhabdomyosarcoma. *Br J Cancer* 1983 48: 261-269.
21. Torres JM, Laborda J, Naval J, Darracq N, Calvo M, Mishal Z, Uriel J. Expression of alpha-fetoprotein receptors by human T-lymphocytes during blastic transformation. *Mol Immunol* 1989 26: 851-857.
22. Uriel J, Faily-Crepin C, Villacampa MJ, Pineiro A, Geuskens M. Incorporation of Alpha-fetoprotein by the MCF-7 Human Breast Cancer Cell Line. *Tumour Biol* 1984 5: 41-51.
23. Jacobson HI, Marotta D, Mizejewski GJ, Bennett JA, Andersen TT. Estradiol-induced changes in spectral and biological properties of alpha-fetoprotein. *Tumor Biol* 1990 11: 104.
24. Mizejewski GJ, Vonnegut M, Jacobson HI. Estradiol-activated alpha-fetoprotein suppresses the uterotrophic response to estrogens. *Proc Natl Acad Sci USA* 1983 80: 2733-2737.
25. Jacobson HI, Bennett JA, Mizejewski GJ. Inhibition of estrogen-dependent breast cancer growth by a reaction product of alpha-fetoprotein and estradiol. *Cancer Res* 1990 50: 415-420.
26. Bennett JA, Mizejewski GJ, Allen SHG, Zhu SJ, Jacobson HI. Transformation of alpha-fetoprotein to a negative regulator of estrogen-dependent growth by ligands of the steroid/thyroid hormone receptor superfamily. *Proc Am Assoc Cancer Res* 1993 34: 244.
27. Festin SM, Fletcher PW, Andersen TT. C-terminal fragment of alphafetoprotein arrests estrogen-dependent growth. *Prot Sci* 1995 4: 111.
28. Line BR, Bennett JA. Scintigraphic Imaging of Human Breast Cancer Xenografts with Tc-99m Recombinant Human Alpha-Fetoprotein. *J Nucl Med* 1996; 37: 87P.
29. Line BR, Bennett JA, Lukasiewicz RL. Rapid Detection of Human Breast Cancer Using Tc-99m Recombinant Human Alpha Fetoprotein and Blood Pool Activity Subtraction. *J Nucl Med* 1996; 37: 148P.
30. Moot SK, Peters GN, Cheek JH. Tumor hormone receptor status and recurrences in premenopausal node negative breast carcinoma. *Cancer* 1987; 60: 382-385.
31. Rosen P, Groshen W, Saigo P, Kinne D, Hellman S. A long-term follow-up study of survival in stage I (T1N0M0) and stage II (T1N1M0) breast carcinoma. *J Clin Oncol* 1989; 7: 355-366..
32. Fisher B, Bauer M, Wickerham L, Redmond C, Fisher E. Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. *Cancer* 1983; 52:1551-1557.
33. Terui S, Yamamoto H. New simplified lymphoscintigraphic technique in patients with breast cancer. *J Nucl Med* 1989; 30: 1198-1204.
34. McLean R, Ege G. Prognostic value of axillary lymphoscintigraphy in breast carcinoma patients. *J Nucl Med* 1986; 27: 1116-1124.
35. Taillefer R, Robidoux A, Turpin S, Lambert R, Benjamin C, Gagnon A, Cantin J, Leveille J. Detection of Axillary Lymph Node Involvement with Tc-99m Sestamibi Imaging in Patients with Primary Breast Cancer. *J Nucl Med* 1996; 37: 75P.

36. Ryu JS, Choi YY, Yang SO, Gong GY, Park JM, Ahn SH, Choi YB, Lee HK. Tc-99m Sestamibi Imaging in the Evaluation of Breast Cancer and Axillary Lymphadenopathy: Comparison of Planar and Spect Imaging. *J Nucl Med* 1996; 37: 252P.
37. Hayes DF. Axillary lymphoscintigraphy for breast cancer: should we do it? Can we do it? [editorial] *J Nucl Med* 1990; 31: 1835-8.
38. Lichtner RB, Julian JA, North SM, Glasser SR, Nicolson GL. Coexpression of Cytokeratins Characteristic for Myoepithelial and Luminal Cell Lineages in Rat 1372NF Mammary Adenocarcinoma Tumors and Their Spontaneous Metastases. *Cancer Res* 1991 51: 5943-5950.
39. Bennett JA, Pilon VA, Briggs DR, McKneally MF. Evaluation of cyclosporine-treated mice as hosts for growing and testing the chemosensitivity of first transplant generation human tumor xenografts implanted under the kidney capsule. *J Natl Cancer Inst* 1985 75: 925-936.
40. Bennett JA, Parnes SM, DeConti RC. Growth and chemosensitivity of human head and neck cancers implanted under the kidney capsule of cyclosporine-immunosuppressed mice. *Ann Otol Rhinol Laryngol* 1989 98: 455-460.
41. Dansereau RN, Line BR. Preparation of dextran 70 injection labeled with technetium 99m for use as a cardiac blood-pool imaging agent. *Am J Hosp Pharm* 1994; 51: 2797-2800.
42. Line BR, Herrmannsdoerfer AJ, Battles AH, Weber PB, Dansereau RN, Blumenstock FA. Premortem biodistribution of radioactivity in the rat: Measurement of blood and tissue activity of tracers used in clinical imaging studies. *Lab Animal Sci* 1994; 44:495-502.
43. Line BR, Neuman PH. Difference Analysis of Antifibrin Images in the Detection of Deep Venous Thrombosis. *J Nucl Med* 1995; 36: 2326-2332.

APPENDICES

Table 3A: Tumor to Thigh Tissue Background Ratio 1 hour

Tumor	AFP	Domain III	Sestamibi	Thallium
BT20	[8] 1.30 (1.28)	[4] 3.59 (2.54)	[7] 0.28 (0.26)	[7] 0.46 (0.53)
DU145	[8] 1.33 (0.58)	[3] 3.00 (1.60)	[5] 0.13 (0.12)	[4] 0.22 (0.13)
LNCaP	[10] 4.32 (1.77)	[5] 2.73 (0.28)	[5] 0.99 (0.61)	[4] 0.38 (0.05)
MCF7	[2] 4.10 (0.01)	[2] 0.55 (0.01)	[5] 0.29 (0.23)	[6] 0.28 (0.22)
MDA	[6] 0.56 (0.20)	[10] 1.57 (1.31)	[6] 0.41 (0.20)	[6] 0.15 (0.11)
MFE	[5] 2.60 (1.09)	[5] 8.50 (6.02)	[4] 0.65 (0.30)	[6] 0.49 (0.22)
MTW9A	[5] 2.95 (0.94)	[4] 4.62 (0.60)	[3] 0.81 (0.22)	[5] 0.65 (0.33)
T47	[2] 0.30 (0.78)	[8] 0.78 (0.47)	[2] 0.11 (0.09)	[3] 0.34 (0.21)

Table 3B: Tumor to Thigh Tissue Background Ratio 24 hrs

Tumor	AFP	Domain III	Sestamibi	Thallium
BT20	[8] 2.26 (2.18)	[4] 4.13 (3.85)	[7] 0.05 (0.07)	[7] 0.09 (0.06)
DU145	[8] 1.65 (1.43)	[3] 1.65 (1.22)	[5] 0.10 (0.06)	[4] 0.26 (0.24)
LNCaP	[7] 3.79 (2.70)	[5] 4.44 (0.82)	[5] 0.97 (0.95)	[4] 0.19 (0.13)
MCF7	[2] 4.56 (2.71)	[2] 1.28 (0.68)	[2] 0.09 (0.04)	[5] 0.20 (0.20)
MDA	[6] 0.93 (0.61)	[12] 1.17 (1.00)	[6] 0.10 (0.08)	[6] 0.23 (0.08)
MFE	[5] 6.04 (1.66)	[5] 10.93 (5.58)	[4] 0.65 (0.39)	[6] 0.61 (0.11)
MTW9A	[5] 3.21 (1.45)	[4] 3.87 (0.75)	[3] 0.63 (0.26)	[4] 0.87 (0.17)
T47	[2] 0.86 (0.49)	[8] 4.62 (2.54)	[2] 0.15 (0.01)	[3] 0.03 (0.02)

Table 3C: Percent Injected Dose per Gram Tumor at 1 hour

Tumor	AFP	Domain III	Sestamibi	Thallium
BT20	[8] 0.98 (0.80)	[4] 2.30 (1.62)	[7] 0.12 (0.08)	[7] 0.33 (0.40)
DU145	[8] 1.16 (0.64)	[3] 1.08 (0.15)	[5] 0.18 (0.16)	[4] 0.48 (0.29)
LNCaP	[10] 3.60 (2.48)	[5] 1.46 (0.09)	[5] 0.49 (0.29)	[4] 0.45 (0.08)
MCF7	[2] 2.25 (0.22)	[2] 0.38 (0.06)	[5] 0.22 (0.14)	[6] 0.36 (0.27)
MDA	[6] 0.27 (0.09)	[10] 0.62 (0.31)	[6] 0.17 (0.09)	[6] 0.21 (0.15)
MFE	[5] 1.11 (0.54)	[5] 2.96 (2.28)	[4] 0.39 (0.19)	[6] 0.34 (0.16)
MTW9A	[5] 2.64 (1.16)	[4] 2.79 (0.30)	[3] 1.31 (0.37)	[5] 1.63 (0.82)
T47	[2] 0.39 (0.85)	[8] 0.39 (0.25)	[2] 0.14 (0.13)	[3] 0.34 (0.18)

Table 3D: Percent Injected Dose per Gram Tumor 24 hours

Tumor	AFP	Domain III	Sestamibi	Thallium
BT20	[8] 0.44 (0.43)	[4] 1.23 (1.11)	[7] 0.01 (0.01)	[7] 0.06 (0.04)
DU145	[8] 2.25 (2.80)	[3] 0.77 (0.67)	[5] 0.06 (0.04)	[4] 0.32 (0.29)
LNCaP	[7] 0.80 (0.48)	[5] 0.93 (0.30)	[5] 0.20 (0.19)	[4] 0.17 (0.13)
MCF7	[2] 2.00 (1.89)	[2] 0.29 (0.16)	[2] 0.03 (0.01)	[5] 0.18 (0.18)
MDA	[6] 0.19 (0.10)	[12] 0.28 (0.16)	[6] 0.01 (0.01)	[6] 0.21 (0.06)
MFE	[5] 1.42 (0.64)	[5] 1.86 (1.52)	[4] 0.12 (0.08)	[6] 0.51 (0.11)
MTW9A	[5] 0.81 (0.58)	[4] 1.75 (0.38)	[3] 0.24 (0.08)	[4] 1.49 (0.19)
T47	[2] 0.29 (0.06)	[8] 3.07 (2.35)	[2] 0.07 (0.00)	[3] 0.04 (0.03)

Data shown as number of animals in brackets, mean and standard deviation in parentheses (see description in body of report).